Vol. 135, No. 2, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 13, 1986 Pages 622-628

ISOLATION AND PRIMARY STRUCTURE OF A PEPTIDE FROM THE CORPORA CARDIACA OF *HELIOTHIS ZEA* WITH ADIPOKINETIC ACTIVITY

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Received January 31, 1986

An adipokinetic hormone was isolated from the corpora cardiaca of the corn ear worm moth, *Heliothis zea*, and purified by reversed phase high performance liquid chromatography. The primary structure, pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2, was determined by automated gas-phase Edman degradation of the peptide deblocked with pyroglutamic aminopeptidase, and by fast atom bombardment mass spectrometry. The hormone was synthesized and the natural and synthetic material had identical chromatographic, spectroscopic, and biological properties. The peptide was found to have lipid mobilizing activity in *H. zea* adults.

In this paper we report the isolation and primary structure determination of the adipokinetic hormone (H-AKH) from the corpora cardiaca of the corn ear worm moth, *Heliothis zea*, a major pest species in the United States. The AKH/RPCH family of peptides from invertebrates contains C- and N-terminally blocked neuropeptides with diverse biological activity in insects (1). To date, the primary structures of seven peptides in this family have been determined (2-6), including the recently reported adipokinetic hormone of *Manduca sexta* (M-AKH)(6). The structure of the latter was determined by a combination of amino acid analysis and fast atom bombardment/tandem mass spectrometry. The primary structure of H-AKH, identical to M-AKH, was determined by automated gas-phase Edman degradation of the deblocked peptide and confirmed by fast atom bombardment-mass spectrometry and by synthesis of the peptide. The synthetic H-AKH had chromatographic, FAB-MS, and UV spectroscopic properties and biological activity identical to the natural hormone.

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Abbreviations Used: AKH, adipokinetic hormone; BR, brain; CA, corpora allata; CC, corpora cardiaca; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography; H-AKH, *Heliothis zea* AKH; M-AKH, *Manduca sexta* AKH; RPCH, red pigment concentrating hormone; SOG, subesophageal ganglion; TFA, trifluoroacetic acid; UV, ultraviolet.

0006-291X/86 $1.50

MATERIALS AND METHODS

Experimental animals. *H. zea* were reared on artificial diet at 26°C, 70% relative humidity and a photoperiod of 16 hours light:8 dark.

Purification of H-AKH. Brain - subesophageal ganglion - corpora cardiaca - corpora allata (BR-SOG-CC-CA) complex or pairs of CC-CA were dissected from 2 - 3 day old adults and placed into 1.5 ml polypropylene centrifuge tubes containing 500 µl chilled methanol:water:acetic acid:thiodiglycol (90:9:1:0.1) and stored at -70°C. Batches of material from 100 - 200 insects were homogenized with a Polytron™ homogenizer (Brinkman Instruments[b]) equipped with a PT 7 microprobe and the homogenate was centrifuged at 4,000 rpm at 5°C for 30 minutes. The pellet was reprocessed by the above procedure and the combined supernatants were transferred to a clean tube and concentrated under a stream of nitrogen to minimal volume. The resulting sample was taken up in 1 ml of 0.1% aqueous TFA and extracted three times with ethyl acetate. Residual ethyl acetate was removed under a stream of nitrogen and the sample was filtered through a Millex-HV™ filter (Millipore).

Method A - Filtered samples were separated using a Supelcosil LC-18DB column with a Pelliguard™ guard column (Supelco) on a Waters Model 840 liquid chromatograph with autosampler. The sample was injected onto the column and eluted with a concave gradient (Waters curve 7) over one hour at 1 ml/min, starting with 10% acetonitrile (0.1% v/v TFA) and 90% aqueous 0.1% TFA, and ending with 60% acetonitrile (0.1% v/v TFA) and 40% aqueous 0.1% TFA. The eluent was monitored spectrophotometrically at 214 nm and by fluorescence at >300 nm (excitation at 230 nm). Fractions were collected at 1 minute intervals with both the autosampler and fraction collector cooled to 0 - 5°C. Fractions with the same retention times from multiple runs were pooled in the fraction collector and those fractions with biological activity (48 - 49 minutes) were combined and lyophilized.

Method B - The active fractions were further purified by reversed phase HPLC on a DuPont Zorbax™ C-8 150 SP column on a Hewlett Packard 1090 liquid chromatograph. The sample was eluted with a linear gradient from 5 to 50% acetonitrile in 0.25 N triethylammonium phosphate, pH 2.20, at 0.4 ml/min. Fractions were collected at 1 minute intervals, the active fractions were pooled, and the acetonitrile was removed *in vacuo*.

Method C - The peptide was finally isolated using the same 1090 chromatograph and Zorbax column, but using a linear gradient from 10 to 50 % acetonitrile (0.1% v/v TFA) against 0.1% aqueous TFA over 1 hour at 0.4 ml/min.

Enzymatic deblocking. Pyroglutamic aminopeptidase (EC 3.4.11.8), 25 units (Sigma) was dissolved in 1 ml of 0.4% N-ethylmorpholinium acetate, pH 7.0. The enzyme solution was aportioned into 100 µl aliquots which were stored at -70°C. Enzyme activity was monitored by digestion of a synthetic H-AKH analog pGlu-Leu-Ser-Phe- Thr-Ser-Trp-Gly-ThrNH_2. To a solution of natural, purified H-AKH (378 ng) in 205 µl N-ethylmorpholinium acetate buffer in a 1.5 ml polypropylene centrifuge tube was added 50 µl (1.25 units) of enzyme solution. The tube was sealed under nitrogen and allowed to stand in the dark for 22 hours at room temperature, after which the deblocked peptide was isolated by reverse phase HPLC using method C.

Amino acid analysis. The amino acid composition of the peptides was determined by amino acid analysis using a Waters Pico-Tag™ system according to procedures supplied by the manufacturer.

Sequence analysis. The purified deblocked peptide (260 pMoles) was sequenced on a Model 470A peptide sequencer (Applied Biosystems) using the 02RPTH program supplied by the vendor. The PTH residues from the sequencer were dried in a Speed-Vac™ concentrator (Savant) and analysed by reversed phase HPLC using a DuPont Zorbax C18 column, eluting with 47% acetonitrile and 53% buffer, consisting of 0.18% aqueous acetic acid adjusted to pH 4.55 with Sequenal grade triethylamine (Pierce), at a flow rate of 1 ml/min, 55°C. The effluent was monitored at 254 nm and the analysis was calibrated with PTH standards (Pierce).

Peptide synthesis. LAKH and the H-AKH analog pGlu-Leu-Ser-Phe-Thr-Ser-Trp-Gly-ThrNH_2 were synthesized commercially (Peninsula). H-AKH was synthesized on a Model 430A peptide synthesizer (Applied Biosystems) using reagents and cycles supplied by the manufacturer. The amino acid used for position one was glutamine, which spontaneously cyclized under the conditions used to affect cleavage from the starting resin (7). The resulting peptide was first purified using a model 1090 chromatograph with a Vydac™ C4 column (Separations Group) with the gradient shown in Table 1. The crude H-AKH eluted at 8.3 minutes under these conditions. The peak material was collected and further purified using method C.

Fast atom bombardment mass spectrometry. FAB-MS was performed on a Kratos MS-50 high field mass spectrometer (8).

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[b]Mention of a commercial product in this paper does not constitute an endorsement by the USDA.

Table 1. Gradient Table for First Purification of Synthetic H-AKH

Time	%A	%B	%C
0	2	94	4
27	7	79	14
30	33.3	0	66.7
35	33.3	0	66.7
37	2	94	4

A is 0.1% (v/v) TFA in acetonitrile, B is 0.1% (v/v) aqueous TFA, C is 0.1% (v/v) TFA in 1-propanol. Flow rate is 1.1 ml/min, 45°C.

Bioassay. Purified natural and synthetic H-AKH and LAKH were dissolved in sucrose-phosphate (0.035 M sucrose, 0.02 M Na_2HPO_4) buffer at 0.5 pmol/µl. The CC-CA dissected from 2 - 3 day old male *H. zea* were homogenized in sucrose-phosphate (1 pair in 10 µl) in a micro tissue grinder (Kontes). The hormone preparation or 1 pair CC-CA were injected into the abdomen of a male *H. zea* during photophase. One hour after the injection, hemolymph (5 µl) was drawn into a capillary tube by puncturing the dorsal aorta in the abdominal region. Total lipids were determined by the vanillin-phosphoric acid method (9) with the use of a commercially available kit (Boehringer Mannheim).

RESULTS AND DISCUSSION

H-AKH was isolated by three gradient elution reversed phase HPLC systems. Fluorescent, tryptophan-containing fractions from the separation of BR-SOG-CC-CA or CC-CA samples were collected at 48-49 minutes using method A and further purified by method B (figures 1A and 1B). The material eluting at 46 minutes was believed to be H-AKH because of its similarity in retention time and spectra to various synthetic AKH/RPCH peptides. Final purification was accomplished using method C (figure 1C). Superposition of the normalized upslope, apex, and downslope spectra indicated peak purity (figure 1D). Purified H-AKH was shown to have lipid mobilizing (AKH) activity in *H. zea*.

Amino acid analysis of purified H-AKH indicated the following composition: Glx(1), Gly(1), Leu(1), Phe(1), Ser(2), and Thr(2). Although tryptophan was not determined by this method, its presence in the peptide

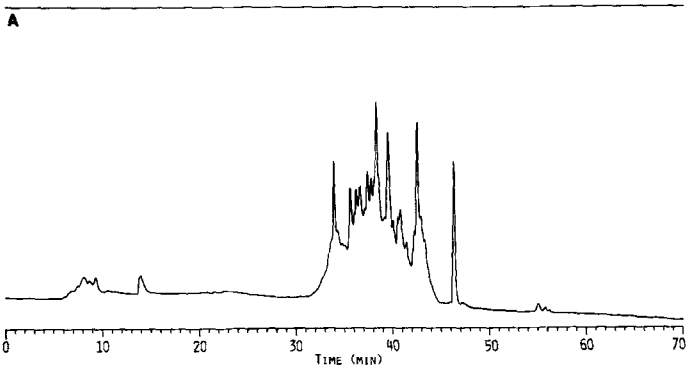

Figure 1A. Elution profile using method B of pooled 48 - 49 minute fractions obtained from approximately 2200 *H. zea* BR-SOG-CC-CA by method A (0.200 A_{210} full scale).

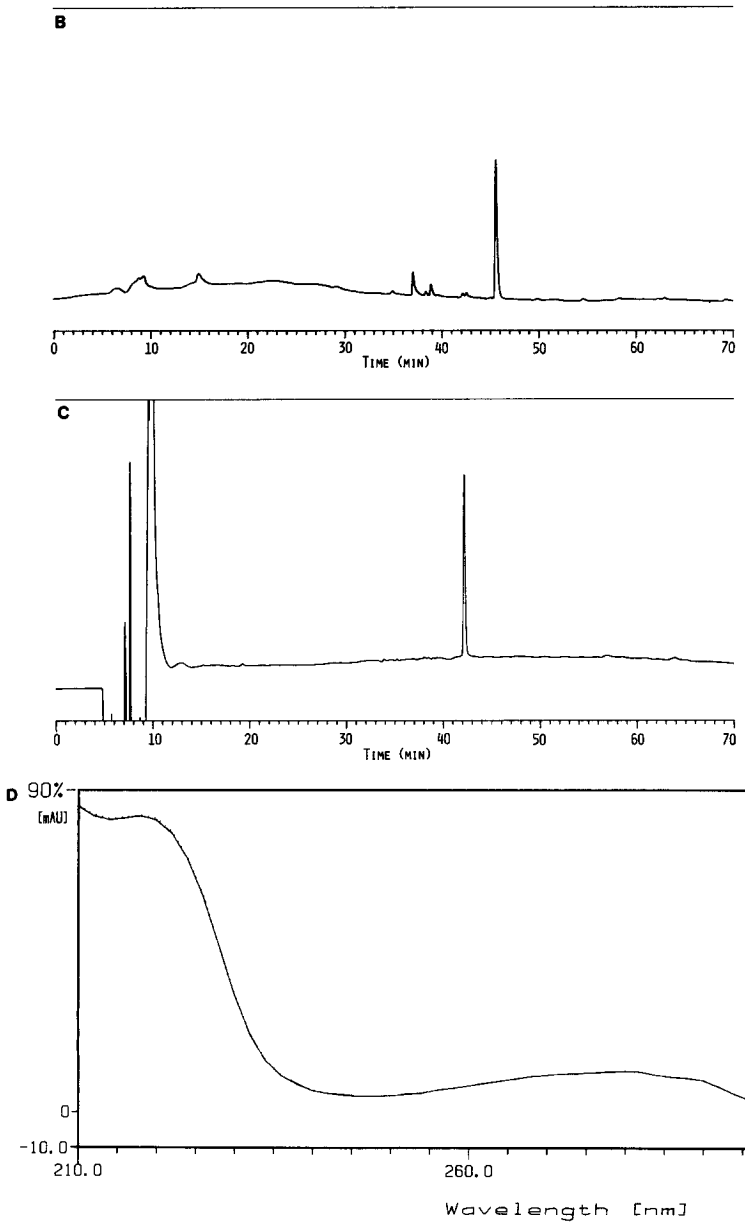

Figure 1B. Elution profile using method B of the 48 - 49 minute fraction obtained from approximately 160 *H. zea* CC-CA by method A (0.100 A_{210} full scale).

Figure 1C. Chromatogram of the elution of 378 pmol of H-AKH using method C (0.200 A_{210} full scale).

Figure 1D. Overlay of normalized UV spectra of the upslope, apex, and downslope of the peak of H-AKH shown in figure 1C.

was shown spectrophotometrically, particularly the second derivative spectrum (figure 2) between 250 and 300 nm (10) and by the difference in mass calculated from the amino acid analysis and the mass measured by FAB-MS. Finally, FAB-MS of H-AKH (figure 3) indicated a molecular weight of 1007, consistent with an amino-terminal pGlu, C-terminal α-carboxamide, tryptophan-containing nonapeptide.

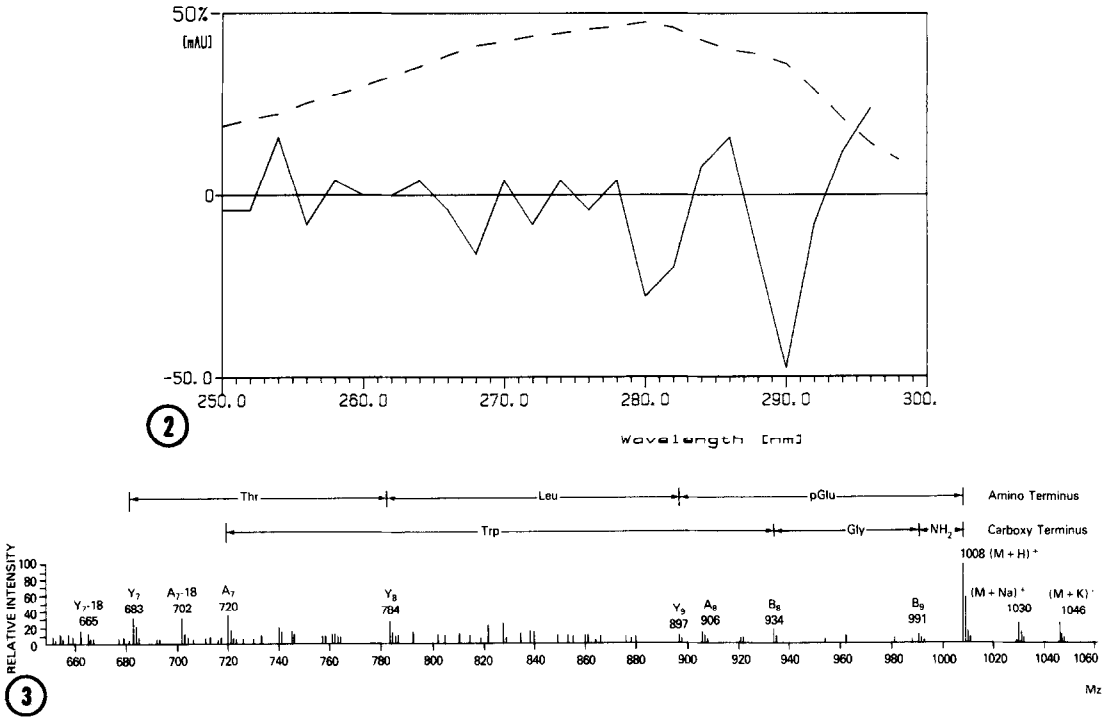

Figure 2. UV spectrum of H-AKH (dashed line) and its second derivative spectrum (solid line) between 250 - 300 nm.

Figure 3. Partial FAB-mass spectrum of *ca.* 250 pmol H-AKH. Nomenclature for fragment ions is that of reference 13.

The primary structure of H-AKH was determined by automated gas-phase Edman degradation of the deblocked natural hormone. The elution profile obtained after digestion of H-AKH with pyroglutamic aminopeptidase (figure 4) displays a major product (H-AKH - pGlu) eluting 4 minutes earlier than H-AKH and a minor product (H-AKH - pGlu-Leu-Thr) eluting at 29 minutes. Photodiode array spectroscopy indicated that both peptides were pure and contained tryptophan.

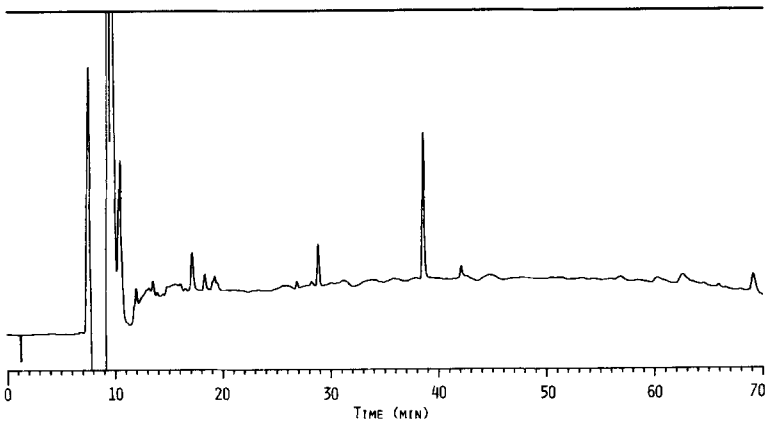

Figure 4. Chomatogram of the elution of the pyroglutamic aminopeptidase digest of 378 pmol H-AKH using method C (0.100 A_{210} full scale).

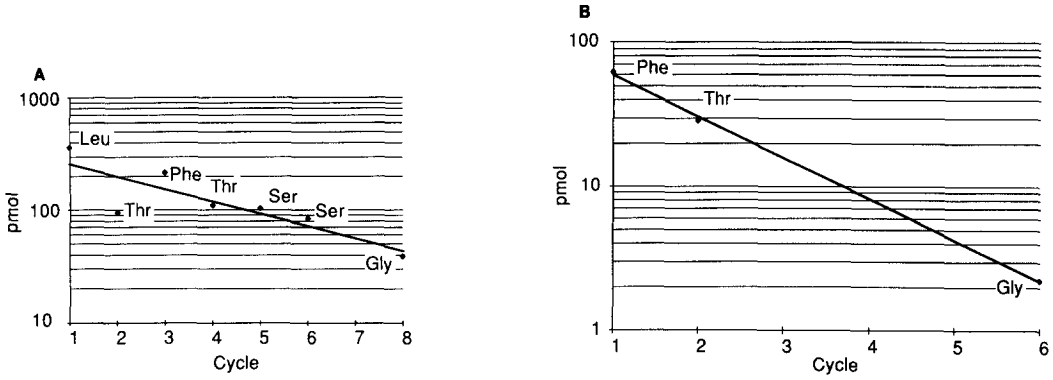

Figure 5. Smithies plot of the sequence analysis of H-AKH: A. 260 pmol H-AKH - pGlu, B. 60 pmol H-AKH - pGlu-Leu-Thr (Ser was observed, but could not be quantitated).

Sequential Edman degradation of H-AKH - pGlu indicated the presence of a single peptide. The data were analysed (figure 5A) by the method of Smithies *et al.* (11) All of the amino acids expected from the amino acid analysis and spectroscopy were found in the PTH analysis with the exception of tryptophan, which was presumed to account for the apparent absence of any residue in cycle seven. The minor product (60 pmol) was sequenced and shown by the same analysis to be a subset of the natural peptide, starting at phenylalanine (figure 5B). Previous work on an H-AKH analog, pGlu-Leu-Ser-Phe-Thr-Ser-Trp-Gly-ThrNH_2, also demonstrated the production of peptide fragments generated by cleavage of the Ser-Phe bond in addition to the pGlu-Leu bond expected (data not shown). The sequence thus obtained was confirmed by synthesizing the peptide. The synthetic peptide was identical to the natural hormone in retention time, UV and FAB-MS spectra. Both natural and synthetic H-AKH caused a significant increase (63%) in the hemolymph lipids (figure 6). The synthetic locust AKH caused a similar increase and confirmed an earlier report of cross reactivity (12).

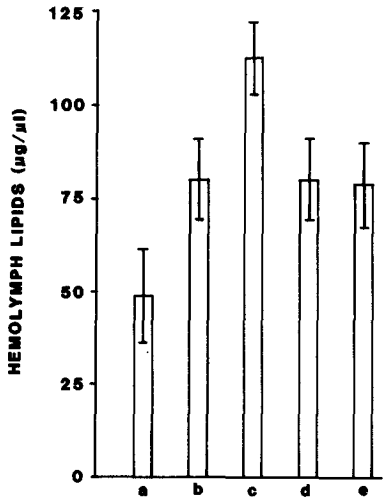

Figure 6. Hemolymph lipids in *H. zea* males one hour after the injection of (a) 10 µl sucrose/phosphate buffer, N = 20; (b) 3.9 pmol natural H-AKH, N = 12; (c) one pair of CC-CA, N = 6; (d) 4.1 pmol synthetic H-AKH, N = 15; and (e) 5 pmol LAKH, N = 9. All means ± S.D.

Table 2. AKH/RPCH family of peptides

	1 2 3 4 5 6 7 8 9 10
LAKH	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-$ThrNH_2$
RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-$TrpNH_2$
CC-1 (M-I,HGHI)	pGlu-Val-Asn-Phe-Ser-Pro-Asn-$TrpNH_2$
CC-2 (M-II,HGHII)	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-$TrpNH_2$
AKHII-S	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-$TrpNH_2$
AKHII-L	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-$TrpNH_2$
H-AKH, M-AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-$GlyNH_2$

The primary structures of the AKH/RPCH family including H-AKH are shown in Table 2. As can be seen, there exists a high degree of peptide sequence homology among members of the family with all members including H-AKH having pGlu -1, Phe-4, and Trp-8, in addition to amidated C-terminals. These invariant residues have suggested both a common ancestral origin (5) of the hormone and the essential nature of these residues in the function of the adipokinetic hormones. The strong homology of H-AKH and M-AKH with CC-2 and human glucagon 5-8 (-Thr-Phe-Thr-Ser-) has been noted previously (6). Although H-AKH has only been evaluated for lipid mobilizing activity in *H. zea*, it may display a range of biological activities associated with the AKH/RPCH family.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of J. Bray, E. Brown, D. Bushman, D. Little, S. Vohra, and K. Young in the maintenance and dissection of the insects and Dr. Yao-Shi Zhang for amino acid analysis.

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